UNIVERSITY OF PENNSYLVANIA

STUDIES OF THE REACTION BETWEEN METHYLENE BLUE AND LIVING CELLS AND TISSUES IN THE TRANSPARENT MOAT CHAMBER INTRODUCED INTO THE RABBIT'S EAR

RICHARD GURLEY ABELL

A DISSERTATION

IN ANATOMY

PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

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STUDIES OF THE REACTION BETWEEN METHYLENE BLUE AND LIVING CELLS AND TISSUES IN THE TRANSPARENT MOAT CHAMBER INTRODUCED INTO THE RABBIT'S EAR [1, 2, 3]

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TWO FIGURES

One of the most fundamental fields in morphology and physiology is that which involves the peripheral blood capillary, the various cells and tissues to which it supplies blood, and the intercellular material which may intervene between the two. In addition to the specific activity of the cells and the constant interchange of substances between them and the blood stream, this field includes the problem of the life story of the cell—its growth, maintenance, and death.

Curiously enough, comparatively little is known of the character of the intercellular substance and its modifications during varying phases of physiological activity. As a corollary our knowledge is equally meager regarding the effect which variations in the intercellular material may have upon the life story of the cell.

[1]This work was aided by a grant made to the Department of Anatomy at the University of Pennsylvania by the Rockefeller Foundation, under which the author held a fellowship. The assistance of a John Lockwood Memorial Traveling Fellowship from Swarthmore College, which was allotted to the author for the session 1932–1933, is also gratefully acknowledged.

[2]Brief reference to some of the results obtained in this study was made by Abell and Clark ('33) at the 1933 Meeting of the American Association of Anatomists.

[3]A dissertation in Anatomy presented to the Faculty of the Graduate School of the University of Pennsylvania in partial fulfilment of the requirements for the degree of doctor of philosophy.

Certainly an important part of this field lies in the intercellular material, for it is this which forms the surrounding medium of the cell. The clearness with which the new growth of blood and lymphatic capillaries, the formation of arteries and veins, the inward migration of fibroblasts, the activity of macrophages, and the development and behavior of several other types of cells and tissues can be observed in transparent chambers inserted into the rabbit's ear, suggested the possibility of observing with the microscope the effect upon such structures of experimental changes in the character of the intercellular substance.

With the purpose of making such studies possible, a type of transparent chamber, known as the moat chamber, was developed by Abell and Clark ('32). This chamber permits the introduction of chemical substances into the intercellular region in a way that does not disturb mechanically the cells and tissues. Since the chamber is transparent, and the layer of cells and vascular tissues within it very thin, microscopic studies can be made of the effect upon the cells and tissues of such chemical modifications of the intercellular substance. Solutions installed within moat chambers can be subsequently removed and quantitative information secured concerning materials taken from and added to them by the living cells and tissues.

Having demonstrated the innocuous character of the installation of Ringer-Locke's solution into moat chambers, methylene blue was chosen for the first chemical studies. Several properties of methylene blue combine to make it particularly well suited for use in such initial experiments. In the first place, many cells stain vitally with methylene blue when in contact with solutions which are sufficiently dilute to be non-toxic. Consequently, under appropriate conditions, the distribution of methylene blue throughout the tissues can be observed following the introduction of non-injurious solutions of this dye into moat chambers. Information can thus be secured of the distance to which methylene blue is able to diffuse through vascular tissue from solutions of known concentration. In the second place, the colorimetric

method for determining the concentration of methylene blue can be used in analyzing solutions withdrawn from moat chambers, following exposure to vascular tissue. In this way the rate of removal of methylene blue by vascular tissue can be determined. In the third place, the ability of methylene blue to exist in a reduced, colorless form, as well as in the oxidized, blue condition, permits observations, in moat chambers, concerning the supply of oxygen by the blood to the tissues.

The present report describes experiments in which the removal of methylene blue by living capillaries and the effect of this dye upon a thin layer of cells and vascular tissue were studied in moat chambers. Data have been secured concerning oxygen diffusion from the peripheral blood capillary, the efficiency of blood capillaries in preventing the general diffusion of methylene blue through the tissue, the rate of removal of methylene blue by the vessels, the kind of stained cells, and the reactions of blood capillary endothelium to various concentrations of methylene blue.

For the opportunity of making these studies, and for many helpful suggestions concerning them, it is a pleasure to express my gratitude to Dr. Eliot R. Clark.

MATERIAL AND METHOD

1. Construction, installation, and care of moat chambers

Details concerning the construction, installation, and care of moat chambers may be obtained from the original description of the chamber by Abell and Clark ('32). Briefly, such a chamber, oval in shape, and composed principally of glass and mica, contains a very thin space called a 'bay,' which communicates at one end, termed the proximal end, with two entrance holes, and at the other end with a reservoir, or 'moat.' Chemical solutions may be placed in the moat, and subsequently removed from it, through two permanently installed access cannulae. Following the insertion of the chamber into a hole cut through the ear of a rabbit, living

tissue, continuous with the subcutaneous tissue of the ear, grows into the bay through the entrance holes. Since the bay, at its distal end, opens directly into the moat, chemical solutions introduced into the moat are free to pass into the bay and there to interact with the living cells and the tissue normally present during and following the vascularization of the chamber (compare figs. 1 and 2). Since moat chambers remain comparatively stable for many months (over a year in one case), and since the volume of the moat is very large compared to that of the tissue in the bay it is possible to conduct experiments which last any desired length of time up to a year or longer.

2. Injections of methylene blue solutions into the moat

a. Injections for which both cannulae were open. In these experiments, methylene blue solutions varying in concentration from 0.02 per cent to 0.05 per cent (1–5000 to 1–2000) were injected into moat chambers, sealed within them, and left for intervals varying in length from several hours to several days. They were then withdrawn, sterile Ringer-Locke's solution being injected into and sealed within the moat at this time. Daily records prepared with the help of a Leitz 'drawing eyepiece,' were made for several days preceding the injection of the methylene blue solution and throughout the experimental period. Photographs were taken to supplement these records, and the tissue was examined for several hours each day with the microscope. For some of these studies the oil immersion lens was used.

In every instance but one, the dye was injected into chambers in which the tissue had not yet grown all the way to the moat, so that before reaching the vessels, it had to diffuse through a region of the bay, between the tissue and the moat, which contained only tissue fluid, emigrated white blood cells, individual fibroblasts, strands of fibrin, cellular debris, and in some cases a few red blood cells.

The methylene blue solutions were prepared by the addition of dry material, secured from Grübler & Co., rectif. nach Ehrlich, to sterile Ringer-Locke's solution. For the technic of injecting these solutions into the moat, sealing them within it, and later removing them, reference should be made to the paper by Abell and Clark ('32) in which these manipulative procedures are described in detail. It is worth noting, however, that at the present time 70 per cent alcohol is used instead of hexylresorcinol for sterilizing the ends of the access cannulae, following their exposure. The alcohol is applied to the ends of the cannulae with a small cotton probe, before removal of the silver plugs, and is not dropped upon the open ends of the cannulae as was previously done with hexylresorcinol. Before the silver plugs are taken out of the cannulae, the alcohol is removed with a small sterile sponge, and the the surfaces of the cannulae allowed to dry completely by evaporation of alcohol. In this way any danger of diluting the contents of the moat with the disinfecting solution is removed. Two Luer syringes with the ends of the needles ground cone-shaped are used each time the moat is opened. With one syringe the moat contents are removed; with the second the moat is filled with the experimental solution, or with Ringer-Locke's solution. Luer syringes sterilized by dry heat are used.

b. Injections for part of which only one cannula was open. In one of the types of experiment to be described, it was necessary to inject the methylene blue solution into the moat with sufficient pressure to lift the top of the chamber slightly above the tissue in the bay. This was done in order to make certain that some of the methylene blue solution reached directly the upper surface of all of the tissue in the bay. When such an injection was to be made, the moat was filled with a solution of methylene blue, and a sterile silver plug placed in one of the cannulae. The tip of the syringe containing the methylene blue solution was then inserted in the open end of the other cannula, and a small additional quantity of methylene blue forced into the moat. Considerable pressure is neces-

sary to accomplish this, and unless some method is used to anchor the tissue in position, it is forced out of the bay. Such a method is the production of masses of precipitate at the periphery of the tissue. This can be accomplished by injecting into the moat a phosphate buffer prepared by adding 39.50 cc. of 0.2M NaOH to 50 cc. of 0.2M KH_2PO_4, sterilized by boiling. A barrier of highly insoluble material will be precipitated in the bay just in front of the most advanced capillaries, 24 to 48 hours following the injection of this solution into the moat. Tissue subsequently grows around these precipitated masses, and is held in the bay by them.

3. Experimental oxygenation of the peripheral cells and tissue in the bay

In order to determine the relation of the oxygen concentration to the distribution of stained cells in the bay between the most advanced capillary and the moat, oxygen was furnished to the cells in this region experimentally. In order to do this, oxygen from a tank containing pure compressed oxygen was passed through the moat, following the removal of the moat contents. Oxygen passed through the moat in this way is absorbed by the fluid in the bay, and readily diffuses to the region of living cells and tissue.

OBSERVATIONS

1. Distribution of stained cells following injections of methylene blue solutions into the moat

a. Distribution following injections during which both cannulae were open. The observations to be reported in this section were made with chambers in which the living, vascular tissue was separated from the moat by a distance which varied in different chambers from approximately 0.50 to 0.75 mm. At the time of the injections, the tissue was growing toward the moat, and microscopic observations made before the insertion of the dye showed the vessels to be normal in behavior and appearance. The distribution of stained cells, based upon a study of five moat chambers, was as follows.

Two to 4 hours after the injection of a 0.05 or a 0.02 per cent solution of methylene blue into the moat, the glass bottom of the bay close to the moat, the masses of leucocytes present in this region, and everything in the bay for a distance of a few micra from the edge of the moat, stain a diffuse, pale greenish-blue. During the next few hours this pale, diffuse color may advance slowly toward the vessels, but no cases have been observed in which the region of such diffuse staining adjacent to the moat increased in width to more than approximately half a millimeter. Six hours following the injection of the dye into the moat, though considerably longer in some cases, living cells which contain pale blue granules can be seen just peripheral but close to the most advanced blood capillary. Twelve to 15 hours later, many leucocytes and a few other types of cells to be subsequently described, contain brilliant blue granules. These stained cells are not distributed evenly throughout the bay, but are located in a narrow zone close to the most peripheral blood capillary. No stained granules have been observed in cells more peripheral to the most advanced capillary than 293 μ, nor, with few exceptions, have any blue granules been seen in cells more proximal to this capillary than 200 μ. Between the region of stained cells peripheral but close to the most advanced capillary, and the diffuse greenish-blue area adjacent to the moat, is a portion of the bay which shows no trace of blue color, in spite of the fact that it contains many living cells, and that methylene blue must diffuse through it in order to reach and to stain the cells close to the peripheral capillary. Two distinct regions of stained material, separated by a third region totally lacking blue color, can thus be observed subsequent to the injection of a 0.02 or a 0.05 per cent solution of methylene blue into the moat. These three regions are shown in figure 1.

There is some variation in different chambers of the distance in front of the most advanced capillary to which living cells stain vitally with methylene blue. In one chamber the most peripheral of such cells was 125 μ beyond the distal capillary—in another chamber 215 μ.

Microscopic examination of the vascular tissue between the most peripheral capillary and the entrance holes shows that as one passes toward the entrance holes the number of intensely stained cells decreases. In some instances there were no deeply stained cells proximal to the most advanced capillary. When such cells were present, their general location was in the region between the distal capillary and the one just proximal to it. It was only comparatively rarely that deeply stained cells were found in the tissue proximal to the second most advanced capillary. Although fairly large numbers of stained cells could be seen for a short distance in the tissue proximal to the second most advanced capillary, they generally contained pale blue rather than intense blue granules.

Not only does the number of intensely stained cells decrease as one passes from the peripheral capillary toward the entrance holes, but the actual number of stained cells decreases as well. At a greater distance proximal to the most advanced capillary than approximately 200 μ the number of stained cells present is very small, and all of them are located at a distance not greater than about 52 μ from venules and veins which form the principal collecting channels for blood from the periphery. Between these vessels the tissue is richly supplied with capillaries, yet contains no stained cells.

Fig. 1 A 'drawing eyepiece' record, showing the distribution of stained cells in a portion of the bay of chamber 2/23/33L, 24 hours following the introduction of a 0.02 per cent solution of methylene blue into the moat. All of the cells shown contained blue granules. Unstained cells are not indicated. The bay opens into the moat at the line BB. The dark region above BB represents methylene blue in a small portion of the moat. The peripheral capillary is located just proximal to 3_A. Sprouts were present on its distal side, but are omitted from this drawing. 1, region of diffuse greenish-blue staining in the bay adjacent to the moat; 2, the colorless region; many living cells were present throughout this region, but did not contain any blue granules; 3, region between the distal capillary and the moat in which many of the cells stained vitally with methylene blue; 3_A, that part of region 3 which was relatively free from debris and fibrin; 3_B, region proximal to the distal capillary in which cells showed concentration of methylene blue. Proximal to 4, only a few cells contained granules stained with methylene blue, and all were located close to venules and veins collecting blood from the peripheral capillary. The irregular line near the left margin of the drawing is the side of the bay. The portion of the bay included in this drawing is indicated in figure 2. × 89.4.

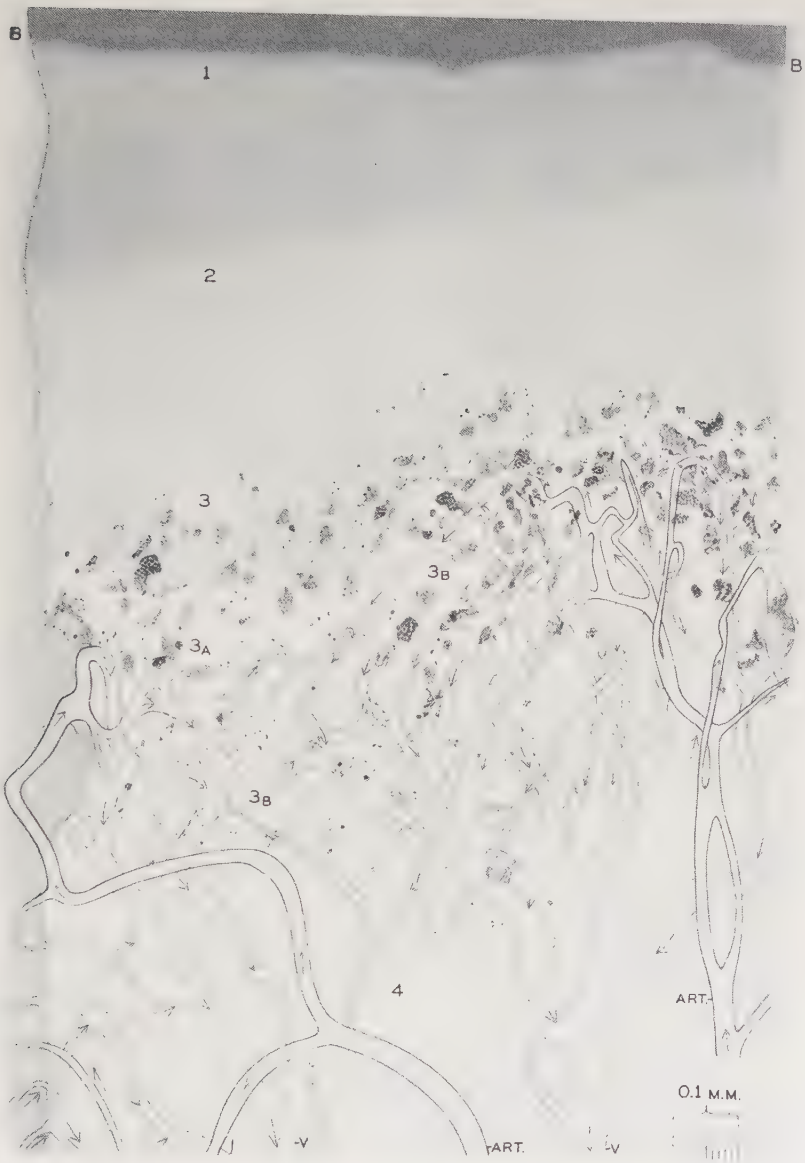

Figure 1

b. The effect of experimental oxygenation upon the distribution of stained cells between the most advanced capillary and the moat. A 0.05 per cent solution of methylene blue was injected into and sealed within the moat of chamber 1/23/32R on November 2, 1932. In this chamber the vessels had grown all the way into the moat, forming a narrow layer of living tissue in the moat at the end of the bay. The vessels of this narrow layer of tissue were injured by the methylene blue solution injected into the moat, so that the circulation through them was stopped. This checked the circulation in the vessels in the bay for a distance of approximately 0.68 mm. from the moat. The most peripheral part of the most advanced capillary containing actively circulating blood three days following the injection of the methylene blue solution was 0.68 mm. from the moat, and at this time a drawing eyepiece record showing the distribution of vitally stained cells in front of it was made. It was observed that, as in the case of the distribution of stained cells between the peripheral capillary and the moat in other chambers, the cells which showed concentration of dye were located in a narrow zone adjacent to the most peripheral capillary which contained actively circulating blood, and that no cells more than 215 μ in front of this capillary contained blue granules. Between the most peripheral of the stained cells and the moat, no trace of methylene blue could be observed, the pale, greenish-blue, diffusely stained region that can usually be seen adjacent to the moat being absent in this case. Following the completion of the drawing eyepiece record, the methylene blue solution was withdrawn, and oxygen passed through the moat. The living cells and tissues were watched continuously with the microscope during and following the passage of oxygen through the moat, and records of changes in the distribution of stained cells were made with the help of a drawing ocular. Details of the experiment are contained in the following protocol:

November 5, 1932, P.M.

4.09. Methylene blue removed from the moat.

410–4.13. Oxygen passed through the moat.

4.10–4.11. Material in the bay adjacent to the moat stained a diffuse greenish-blue for a distance which varied in different parts of the bay adjacent to the moat from approximately 160 to 225 μ. The typical colorless region between this greenish-blue stained area and the most peripheral of the stained cells close to the most advanced capillary could be clearly seen at this time.

4.20. Pale blue granules began to appear in some of the cells located in the originally colorless region, at its peripheral edge.

4.25. Stained granules appeared in more cells near the periphery of the region which had previously been colorless, and the depth of color deepened somewhat in those cells which first showed blue granules following the passage of oxygen through the moat.

4.27–4.30. Oxygen passed through the moat. The staining of cells gradually advanced toward the vessels, and those in which the blue color first appeared became more deeply stained.

5.00–5.45. Oxygen passed through the moat. The color progressively deepened in the newly stained cells, until it became as brilliant as in those close to the vessels which were stained before administration of oxygen was begun. The colorless region separating the newly stained cells from those close to the vessels gradually became narrower. At 5.45 the entire region between the greenish-blue diffusely stained area adjacent to the moat, and the region of original staining close to the most advanced capillary, was almost as completely filled with cells stained a brilliant blue as was the narrow zone close to the peripheral capillary which contained stained cells before oxygen was passed through the moat.

6.00. Ringer-Locke's solution injected into and sealed within the moat

November 7, 1932, P.M.

1.45. The chamber was studied with the microscope. At this time the greenish-blue, diffusely stained region close to the moat was still present, and had not changed in appearance nor receded toward the moat. The cells in which blue granules appeared following passage of oxygen through the moat showed no trace of dye, but the cells in the original zone of staining close to the peripheral capillary contained brilliant blue granules.

c. Distribution of stained cells in the tissue proximal to the most advanced capillary, following injections of methylene blue during part of which only one cannula was open. Previous to the time of injection of methylene blue solutions into moat chambers, it had been supposed that after such injections the dye, upon reaching the peripheral margin of the living tissue, would gradually diffuse proximally through this tissue until it reached the entrance holes, at which time stained cells would be present throughout the entire extent of tissue in the bay. This did not turn out to be the case, however, the stained cells in the living tissue proximal to the most advanced capillary being limited in distribution to a narrow zone close to the peripheral capillary and to the principal vessels collecting blood from the periphery. It seemed desirable to determine whether this localization of stained cells was due to removal of the dye by the most advanced capillaries, which thus prevented it from reaching the proximal tissue, except by diffusion from the vessels which collected the blood from the periphery, or whether methylene blue actually did diffuse past the most advanced capillaries, but could not be seen in the more proximal tissue because of reduction. In order to find out which of these alternatives was the correct one, it was necessary to make certain that some of the methylene blue solution reached all of the tissue in the bay. This was accomplished by injecting the methylene blue solution into the moat with sufficient force to lift the mica top of the bay slightly above the tissue, thus permitting some of the dye to come in direct contact with the upper surface of all of the tissue in the bay. Twenty minutes following such an injection of 0.05 per cent solution of methylene blue into chamber 2/17/32L, in which the peripheral edge of the tissue was anchored by masses of precipitate, produced by a temporary installation in the moat of a phosphate buffer, many vitally stained cells could be seen throughout the entire extent of tissue in the bay. Large numbers of cells were stained in the proximal tissue all the way to the entrance holes, and, although most of these cells were located close to

blood vessels, there was no restriction of their distribution to the zone adjacent to the peripheral capillary or to the capillaries, venules, and veins draining blood from the periphery.

2. Description and classification of stained cells.

Many of the stained cells could be identified by their size and behavior as macrophages. A few of the adventitial cells, most of which were located along the more peripheral capillaries, but a small number of which were present in the more proximal tissue close to venules and veins collecting blood from the periphery, contained relatively large, pale, round, homogeneously stained structures. Definite, round, homogeneously stained structures were likewise observed in some, but not all, of the endothelial cells of the peripheral capillaries, and in a rather smaller proportion of the endothelial cells of the venules and veins draining blood from the periphery. Concentration of dye by macrophages was relatively lasting, in contrast to the temporary character of staining of adventitial and endothelial cells.

Beside the previously mentioned cells which could be definitely identified, many cells containing stained granules arranged in more or less regular rows were seen. Many deeply stained cells, smaller than macrophages, but probably leucocytes, were observed in the region peripheral to the most advanced capillary. In several instances leucocytes containing blue granules were observed within blood capillary sprouts and within blood capillaries containing actively circulating blood.

3. The rate of removal of methylene blue by the blood capillaries

Since the moat, as well as the bay, has a glass bottom and a mica top, marked variations in the depth of color of a methylene blue solution within it can be detected with the naked eye. During the 12 days that a 0.020 per cent solution of methylene blue was left in the moat of chamber 2/23/33L, daily

macroscopic observations showed that its color gradually became lighter. On the twelfth day, the color of the methylene blue solution within the moat was decidedly lighter than it had been when the solution was first placed in the moat.

In one case an attempt was made to determine colorimetrically the exact amount of methylene blue absorbed from the moat. Twelve days after the 0.020 per cent solution of methylene blue was injected into the moat of chamber 2/23/33L, a chamber which did not contain any lymphatic vessels, the moat was opened, its contents removed, and the concentration of methylene blue determined by comparison in a Spencer Duboscq colorimeter with a standard methylene blue solution. Two series of readings were made, the first immediately following the removal of the solution from the moat, the second 40 minutes later. A comparison of the results secured from these two series showed that the methylene blue solution did not change in color during this time, and that during the 12 days that the 0.02 per cent (1–5000) solution of methylene blue was left in the moat it decreased in concentration from 0.020 per cent to 0.003 per cent. Consequently, 85 per cent of the methylene blue present in the moat at the time the 0.020 per cent solution was sealed within it was removed in 12 days. Figure 2 is a photomicrograph of vessels and other structures in the bay of chamber 2/23/33L.

4. Removal of methylene blue by the lymphatics

Chamber 2/23/33L contained no lymphatic vessels, so that none of the methylene blue removed from the moat in this chamber could be attributed to lymphatic participation. A 0.05 per cent (1–2000) solution of methylene blue has been injected, however, into three chambers which contained both blood and lymphatic capillaries. In none of them did any of the leucocytes within the lymphatics show concentration of the dye, nor were any blue stained structures observed within cells which formed the walls of the lymphatics, or in cells near lymphatics. In every instance the lymphatics ended at least 200 μ behind the most advanced blood capillaries,

which thus intervened between them and the source of the dye. The distribution of stained cells in chambers containing both lymphatic and blood vessels was similar to their distribution in chambers that contained only blood vessels. There is thus no evidence from these studies to show that the lymphatics played any part in removal of the dye.

Fig. 2 A photomicrograph, showing blood vessels and other structures in the bay of chamber 2/23/33L 8 days following the installation of a 0.02 per cent solution of methylene blue into the moat. Brackets E and E_1 are placed below the edges of the two entrance holes through which the new tissue invaded the bay. The bay extends from the line D to the line BB. The arrows pointing upward are placed next arterioles, A; those pointing downward next veins, V. The portion of the bay shown at higher magnification in figure 1 is that included within the rectangle at the upper left corner. The legends for 1, 2, 3, 3_A, 3_B and 4 are identical with those given for the same designations in figure 1. × 17.5.

5. Reaction of blood capillary endothelium to methylene blue.

The injection of a 0.5 per cent solution of zinc free[4] methylene blue into the moat of chamber 6/14/33R caused severe hemorrhage, within 30 minutes, of all of the vessels in the bay for a distance of 0.4 mm. The installation of 0.05 per cent solution of methylene blue into chamber 1/23/32R, destroyed blood capillaries present in the moat at the time of injection of the dye. A slight hemorrhage of the most advanced capillary loops in the bay of chamber 7/30/31L was produced by a 0.05 per cent solution of methylene blue, sealed within the moat and left overnight. In other instances the injection of a 0.05 per cent solution into the moat did not produce hemorrhage of any of the vessels in the bay. The injection of a 0.02 per cent solution of zinc free[4] methylene blue into the moat of chamber 2/23/33L destroyed one peripheral capillary loop, but not others adjacent to it. Staining of the nuclei of the endothelial cells of this particular capillary loop preceded its destruction. Why one capillary loop should have been destroyed but not others near it, is unknown.

In some cases sticking of leucocytes to the endothelium developed in the peripheral capillary and in the venules and veins collecting blood from the periphery, following the introduction of a 0.05 per cent solution of methlene blue into the moat. In other instances a methylene blue solution of the same concentration produced no such sticking. A 0.003 per cent solution of methylene blue present within the moat of chamber 2/23/33L produced no visible toxic effect upon the vessels in the bay.

[4]For analysis of a sample of the methylene blue used in this experiment I am indebted to Dr. John H. Muller, professor of chemistry in the Graduate and Towne Scientific Schools of the University of Pennsylvania.

DISCUSSION

The distribution of cells showing concentration of methylene blue in the region between the most advanced blood capillaries and the moat, and the presence, distal to such stained cells, of a portion of the bay in which no trace of blue color can be detected appears to be consistent with the view that as the methylene blue diffuses from the moat into the bay, it undergoes alternate reduction and oxidation. According to this conception, the methylene blue is changed to leucomethylene blue as it passes into the region of living cells removed from the blood capillaries by a distance greater than approximately 293 μ. When the leucomethylene blue enters the region of higher oxygen concentration close to the blood capillaries, it is oxidized within many of the cells, possibly also outside of them, to methylene blue.

That living tissue is able to reduce methylene blue has been demonstrated by the studies of Ehrlich (1885 and 1886), Arnstein (1887), Underhill and Closson ('05) and others. That reduced methylene blue actually was present in the colorless region of the bay, following the introduction of a solution of methylene blue into the moat, is demonstrated by the appearance of brilliant blue granules in many of the cells in this region, subsequent to experimental increase of the oxygen concentration.

The data indicate the presence of a gradient of oxygen concentration at the periphery of vascular tissue in moat chambers.

The present observation that a 0.05 per cent solution of methylene blue, introduced into moat chambers, does not diffuse quickly throughout the tissue in the bay, is interesting in connection with observations described by E. R. and E. L. Clark ('33) concerning the character of the intercellular substance. These investigators present evidence that in living subcutaneous tissue in 'round-table' chambers introduced into the rabbit's ear, free fluid is not present outside of the lymphatic and blood vessels. This appears also to be true of the vascularized tissue in moat chambers. Although the oscil-

lation of extravasated blood cells indicating free fluid can frequently be seen in the unvascularized area during the first few days of growth of new vessels and connective tissue into moat chambers, this movement ceases above and between the tissue cells with the progressive vascularization of the chamber. Bobbing back and forth of blood cells can sometimes be observed between the peripheral capillary and the moat even during later stages of ingrowth, but the material above the vascular tissue loses its fluid character. So also does that between the tissue cells. If free fluid were actually present outside of the blood and lymphatic vessels, or between the tissue and the mica cover of the bay; it appears likely that more general diffusion of methylene blue would occur.

The experiments of Ehrlich (1885 and 1886) show that tissues containing reduced methylene blue acquire a blue color upon exposure to air, due to oxidation of the leucomethylene blue into the blue product. Underhill and Closson ('05) demonstrated the excretion of methylene blue in the urine in the form of leucomethylene blue (salt) as well as in the form of methylene blue, and showed that such methylene blue urine gradually assumes a deeper color upon exposure to air for 25 to 30 minutes. If the urine is made alkaline, the change in color occurs at once, due to formation and rapid oxidation of leucomethylene blue (base).

There is a possibility that the gradual change in depth of color of a methylene blue solution sealed within the moat, with free access to the blood vessels in the bay, may have been due to partial reduction of the methylene blue by substances diffusing into the moat from the living tissue in the bay. Assuming that this may have been the case, it appears evident that no methylene blue in the solution removed from the moat was present in the reduced form at the time its concentration was colorimetrically determined, since no change in color of the solution occurred during exposure to the air for 40 minutes. This length of time should have been sufficient to cause a change in depth of color even had the reduced methylene blue been present in the form of the salt.

The colorimetric determination of the concentration of methylene blue in the solution removed from the moat 12 days following its injection into the chamber is believed, therefore, to show, within the limits of experimental error of the method, the actual concentration of methylene blue in the solution at this time, and consequently to demonstrate that a solution of methylene blue, injected into and sealed within the moat, with free access to living blood capillaries in the bay, undergoes decrease in concentration. Under the conditions present during this experiment, 85 per cent of the methylene blue present at the time of injection of the methylene blue solution into the moat was removed in 12 days.

Staining of granules in endothelial cells of the peripheral capillaries and of the venules and veins collecting blood from the periphery, and the appearance of blue granules in adventitial cells and macrophages located in such positions in the proximal tissue that methylene blue was able to reach them only by rediffusion out of the vessels collecting blood from the periphery, show that methylene blue actually entered the peripheral blood capillaries in the bay. The distribution of stained cells in the tissue proximal to the most peripheral capillary indicates that most of the methylene blue was removed by way of the two most advanced rows of capillaries, the greater part probably by way of the capillary closest to the source of the dye, that is, the most peripheral capillary.

Direct passage of methylene blue through the walls of the blood vessels following injection of solutions of this dye into serous cavities of the dog was reported by Starling and Tubby (1894). Landis ('26) observed that certain dyes (including methylene blue) pass through the walls of the venous capillary almost as easily as through simple endothelium, while Clark, Clark and Williams ('34) demonstrated that methylene blue, following intravenous injection into vessels of the rabbit's ear, is able to pass through the walls of veins as well as through those of the capillaries. In this connection the experiments of Shipley and Cunningham ('16), who reported the active absorption of potassium ferrocyanide and iron ammonium

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